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α-AMINOADIPATE AMINOTRANSFERASE OF RAT LIVER MITOCHONDRIA

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SUMMARY

α-Aminoadipate aminotransferase was partially purified from rat liver mitochondria. The enzyme catalyzes a reversible transamination between α-aminoadipate and α-ketoglutarate. The aminotransferase has rather loosely bound coenzyme; a prolonged dialysis of enzyme against phosphate buffer resulted in an almost complete loss of its catalytic activity. The enzymic activity of the apoenzyme was largely restored by either pyridoxal or pyridoxamine phosphate. The equilibrium constant of the reaction was about 1.32 at pH 7.5 and at 37°, and the Michaelis constants for α-aminoadipate, glutamate, α-ketoadipate, and α-ketoglutarate were 9.0, 5.0, 0.5 and 1.3 mM, respectively.

α-Aminoadipate aminotransferase has a strict substrate specificity. In transamination with α-ketoglutarate, α-aminopimelate and norleucine were about 14% and 15% active in place of α-aminoadipate, whereas the other amino acids tested were inert as substrates.

INTRODUCTION

α-Aminoadipate has been implicated as an intermediate of lysine catabolism in mammalian tissues[1]. Recent investigations with rat and human livers have indicated that lysine is converted to saccharopine (ε-*N*-(L-glutaryl-2)-L-lysine)[2–4] and subsequently to α-aminoadipate[5], and it is suggested that this may represent a major degradative pathway of lysine in mammalian liver. Since the transformation of lysine to α-aminoadipate *via* this pathway is catalyzed by enzymes localized in the mitochondria[2,5], a study was undertaken to clarify the mitochondrial metabolism of α-aminoadipate. Preliminary studies have shown that α-aminoadipate is converted, in the mitochondria, to α-ketoadipate by transamination with α-ketoglutarate. The present report provides evidence that this reaction is catalyzed by a specific transaminase, distinct from aspartate aminotransferase or other known transaminases,

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and also describes some of the properties of the enzyme. The enzyme responsible for the transamination of α-aminoadipate will be referred to as α-aminoadipate aminotransferase.

EXPERIMENTAL PROCEDURE

All chemicals were obtained from commercial sources. Brushite was prepared by slowly admixing 0.5-M solutions of Na_2HPO_4 and $CaCl_2$. Glutamate dehydrogenase was prepared from acetone powder of beef liver by the method of STRECKER[6], and crystalline aspartate aminotransferase purified from pig heart mitochondria was a gift from Dr. T. Watanabe, Department of Biochemistry, Osaka University School of Medicine.

Albino rats of Wistar strain weighing approx. 200 g were used in the present investigation. The animals were fasted for 24 h prior to sacrifice, and they were killed by decapitation. The freshly excised livers were homogenized in 0.25 M sucrose, and the mitochondrial fraction was obtained by centrifugation in the usual manner[7]. The mitochondrial pellets were washed once with the sucrose solution and were finally suspended in a small volume of 0.05 M potassium phosphate buffer (pH 7.5), containing 1 mM EDTA. The suspension could be stored at −30° for at least 4 months without appreciable loss of α-aminoadipate aminotransferase activity.

Protein was estimated by the method of LOWRY *et al.*[8] with bovine serum albumin as the standard. High-voltage paper electrophoresis was performed for 60 min at 2000 V in a buffer consisting of pyridine–acetic acid–water (1:10:289, by vol.) on Toyo Roshi No. 2 filter paper. After separation the filter paper was thoroughly dried and immersed in 0.25% ninhydrin in acetone. Amino acids were visualized by heating the electrogram at 60° for 10 min.

Assay of enzyme activity

α-Aminoadipate aminotransferase catalyzes a reversible transamination according to Eqn. 1,

$$\alpha\text{-Aminoadipate} + \alpha\text{-ketoglutarate} \underset{b}{\overset{a}{\rightleftharpoons}} \alpha\text{-ketoadipate} + \text{glutamate} \qquad (1)$$

The α-aminoadipate aminotransferase activity could be measured from either direction (Reaction 1a or 1b) by the rate of disappearance or formation of α-ketoglutarate.

Assay 1. Assay with glutamate and α-ketoadipate as substrates (Reaction 1b). The standard assay mixture contained, in a volume of 0.3 ml, 50 μmoles of potassium phosphate buffer (pH 7.5), 20 μg of pyridoxal phosphate, 0.5 μmole of α-ketoadipate, and an appropriate amount of enzyme. After a 5-min incubation at 37°, the reaction was started by addition of 0.2 ml of 0.1 M potassium L-glutamate and was usually allowed to proceed for 10 min at 37°. The reaction was terminated by adding 0.1 ml of 1 M HCl. After neutralization of the mixture with 0.1 ml of 1 M KOH, an aliquot (routinely 0.3 ml) was determined for α-ketoglutarate. α-Ketoglutarate was estimated by the amount of NADH oxidized in the presence of NH_4^+ and glutamate dehydrogenase. The estimation of α-ketoglutarate was performed in a system consisting of 300 μmoles of potassium phosphate buffer (pH 7.5), 150 μmoles of NH_4Cl, 0.3 μmole

of NADH and the neutralized reaction mixture in a total volume of 3.0 ml. The decrease in absorbance at 340 mμ which occurred on addition of glutamate dehydrogenase was measured in a Hitachi UV-VIS spectrophotometer Model 139. The equilibrium of glutamate dehydrogenase reaction which is far toward the glutamate formation enabled quantitative determination of α-ketoglutarate under these conditions. α-Ketoadipate and glutamate present in the reaction mixtures did not interfere with the determination. α-Aminoadipate aminotransferase reaction, carried out under the standard assay conditions, was linear with respect to time and enzyme concentration until 0.1 μmole of α-ketoglutarate was produced.

Assay 2. Assay with α-aminoadipate and α-ketoglutarate as substrates (Reaction 1a). The reaction mixture was the same as for Assay 1 except that α-ketoadipate and glutamate were replaced by α-ketoglutarate and L-α-aminoadipate. The α-ketoglutarate remaining in the reaction mixture was estimated as above and the amount of the compound which disappeared was calculated by subtracting this value from the suitable control.

Purification of enzyme

All operations were carried out at 0–5°.

Step 1. A suspension of mitochondria was diluted with distilled water to give a protein concentration of about 20 mg per ml. To the suspension were added 0.3 vol. of 0.5 M potassium phosphate buffer (pH 6.5) and 0.4 vol. of 0.1 M adipate. The suspension was also made 1 mM and 8 μM with respect to EDTA and pyridoxal phosphate, and the mixture was heated in a water bath at 53° for 3 min. After rapid cooling, the denatured protein was discarded by centrifugation.

Step 2. To 350 ml of the clear supernatant solution from Step 1 were added 87.5 g of solid $(NH_4)_2SO_4$; the pH was held at about 7 by dropwise addition of 10% KOH. After 30 min, the precipitate was discarded by centrifugation, and 70 g of $(NH_4)_2SO_4$ were added to the supernatant. The resulting precipitate was collected by centrifugation, and was dissolved in a minimal volume of 0.05 M potassium phosphate buffer (pH 7.5), which also contained 1 mM EDTA and 8 μM pyridoxal phosphate. The enzyme solution was then dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7.5)–1 mM EDTA–8 μM pyridoxal phosphate.

Step 3. The dialyzed enzyme solution from Step 2 was poured over a column (2 cm × 19 cm) of DEAE-cellulose which had been equilibrated with 0.01 M potassium phosphate buffer (pH 7.5). The α-aminoadipate aminotransferase activity was not tightly held by the cellulose under these conditions and was eluted just behind the first protein peak. The fractions containing α-aminoadipate aminotransferase activity were pooled and the combined eluate was concentrated by precipitation with $(NH_4)_2SO_4$.

Step 4. The concentrated enzyme solution from Step 3 was dialyzed against 0.05 M potassium phosphate buffer (pH 6.8)–1 mM EDTA–8 μM pyridoxal phosphate, and was applied to a 2 cm × 11 cm column of brushite, which had previously been equilibrated with the same buffer. After washing the column with 120 ml of the buffer, the aminotransferase activity was eluted with 0.09 M potassium phosphate–1 mM EDTA–8 μM pyridoxal phosphate buffer at pH 6.8. The fractions having high specific activity were combined and diluted 2-fold with water. The mixture was then

concentrated by adsorption on a short column of brushite and elution with a strong buffer (0.2 M).

The procedure resulted in a purification of approx. 100-fold with an overall recovery of about 23%. A summary of the purification procedure is presented in Table I.

TABLE I

PURIFICATION OF α-AMINOADIPATE AMINOTRANSFERASE FROM RAT LIVER MITOCHONDRIA

Enzyme activity was measured by Assay 1. A unit of enzyme activity was defined as the amount of enzyme producing 1 μmole of α-ketoglutarate per min under the standard assay conditions, and specific activity was expressed as the number of units per mg of protein.

	Volume (ml)	*Protein content (mg/ml)*	*Total units*	*Specific activity*	*Yield (%)*
Mitochondrial suspension	120	45.0	108	0.02	
Heat treatment	350	4.6	103	0.06	95
$(NH_4)_2SO_4$	6.4	45.6	66	0.14	61
DEAE-cellulose and $(NH_4)_2SO_4$	2.0	34.0	51	0.75	47
Brushite	5.0	2.4	25	2.05	23

RESULTS

Products of reaction

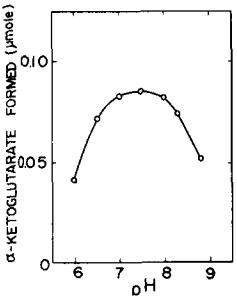
2 μmoles each of α-ketoadipate and L-glutamate were incubated with α-aminoadipate aminotransferase (Step 4) in 1 ml of 0.1 M potassium phosphate buffer (pH 7.5), containing 40 μg of pyridoxal phosphate. The reaction was terminated by addition of 0.2 ml of 1 M HCl, and an aliquot was determined for α-ketoglutarate by glutamate dehydrogenase. Another aliquot (0.4 ml) of the mixture was put on a column, 1 cm × 3 cm, of Dowex-50, H^+ form. After washing the column with 10 ml of water, the amino acids were eluted with 6 ml of 2 M pyridine. The effluent was then taken to dryness under reduced pressure, and the residue dissolved in water. Electrophoresis carried out on a suitable aliquot of the above mixture revealed two ninhydrin-positive spots indistinguishable from authentic α-aminoadipate and glutamate. The spots corresponding to these amino acids were eluted with 4 ml of 50% ethanol solution, and the absorbances of the eluates were determined at 570 mμ. Table II shows the stoichiometry and requirements for the reaction performed under these conditions. The α-aminoadipate aminotransferase reaction was reversible. Incubation of α-aminoadipate and α-ketoglutarate resulted in a formation of glutamate.

pH optimum

α-Aminoadipate aminotransferase was most active at pH 7.5, when assayed in the reverse direction (Reaction 1b) in 0.1 M potassium (pyro)phosphate buffer (Fig. 1).

Coenzyme requirements

With crude mitochondrial extract, addition of pyridoxal phosphate (0.01 mM)



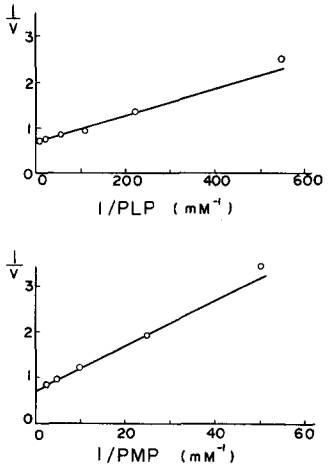


Fig. 1. α-Aminoadipate aminotransferase activity as the function of pH. Activity measurements were made by Assay 1 with 5 μg of Step-4 protein.

Fig. 2. Effect of pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP) concentrations on activation of the apoenzyme. The apoenzyme (10 μg) was preincubated with either pyridoxal or pyridoxamine phosphate for 5 min at 37°. The enzymic activity was measured by Assay 1.

increased the reaction rate 3–4-fold. Dialysis of a purified enzyme against 0.1 M potassium phosphate buffer (pH 6.8), containing 1 mM EDTA, resulted in almost complete loss of its catalytic activity, which was restored to an extent of about 80% of the original activity by adding pyridoxal phosphate. The dialyzed preparation was also activated by pyridoxamine phosphate. Fig. 2 shows the effect of pyridoxal and pyridoxamine phosphates on the activity of the apoenzyme. Apparent dissociation constants of 4.4 and 63 μM were calculated for pyridoxal and pyridoxamine phosphates, respectively.

Equilibrium constant

The equilibrium of reversible α-aminoadipate aminotransferase reaction was examined from both forward and reverse directions (Reactions 1a and 1b). Since the enzyme catalyzes the stoichiometric conversion of amino and keto substrates

TABLE II

STOICHIOMETRY AND REQUIREMENTS FOR TRANSAMINATION BETWEEN α-KETOADIPATE AND GLUTAMATE

Experimental conditions are described in the text.

System	*α-Keto-glutarate (μmole)*	*α-Amino-adipate (μmole)*	*Glutamate (μmole)*
Complete	+0.41	+0.42	−0.44
−enzyme	+0.006		
−α-ketoadipate	0		
−glutamate	0		

(Table II), the final concentration of α-ketoglutarate in the reaction mixture was measured. An average value of 1.32 was calculated for the apparent equilibrium constant,

$$K_{eq} = \frac{[\alpha\text{-ketoadipate}]\ [\text{glutamate}]}{[\alpha\text{-aminoadipate}]\ [\alpha\text{-ketoglutarate}]},$$

at pH 7.5 and at 37° (Table III).

TABLE III

EQUILIBRIUM CONSTANT

The reaction mixture contained, in a volume of 3.0 ml, 500 μmoles of potassium phosphate buffer (pH 7.5), 120 μg of pyridoxal phosphate, 20 μg of Step-4 protein, and amino acid and keto acid substrates as indicated. Incubation was at 37°. At time intervals, 0.5-ml aliquots were removed from the reaction mixture for α-ketoglutarate determination in order to assure the point of equilibrium.

Initial concn. (mM)				*Final concn. (mM)*	K_{eq}
α-Amino-adipate	*α-Keto-glutarate*	*Glutamate*	*α-Keto-adipate*	*α-Keto-glutarate*	
0.500	0.500			0.234	1.29
0.500	0.267			0.080	1.40
0.267	0.500			0.304	1.81
0.267	0.267			0.126	1.23
		0.500	0.500	0.252	1.03
		0.500	0.267	0.186	1.38
		0.267	0.500	0.180	1.24
		0.267	0.267	0.138	1.16
				Average	1.32

Kinetic analysis

The results of initial velocity studies for glutamate–α-ketoadipate reaction, in which the concentration of one substrate was varied at several fixed concentrations of the other are shown in Figs. 3 and 4. Plots of reciprocals of initial velocities against reciprocal concentrations of the other substrate yielded a family of parallel lines. The data depicted in the figures are consistent with the mechanism proposed for other transaminases, involving a single binary enzyme–substrate complex[9,10] and may be represented by the equation,

$$\frac{E_0}{v} = \frac{1}{V}\left(\frac{K_{(\alpha\text{-ketoadipate})}}{[\alpha\text{-ketoadipate}]} + \frac{K_{(\text{glutamate})}}{[\text{glutamate}]} + 1\right)$$

where v represents initial volicity; V, maximum velocity; and $K_{\alpha\text{-ketoadipate}}$ and $K_{\text{glutamate}}$, their Michaelis constants. The Michaelis constants were calculated from the secondary plots of reciprocals of apparent maximum velocities *vs.* reciprocals of concentrations of the other substrate. Values of 0.5 and 5.0 mM were found for α-ketoadipate and glutamate, respectively. Similar kinetic analysis for α-aminoadipate–α-ketoglutarate reaction gave Michaelis constants of 9.0 and 1.3 mM for α-aminoadipate and α-ketoglutarate, respectively. As is the case with aspartate and alanine aminotransferases, the keto substrates have greater affinity for the enzyme[9,10].

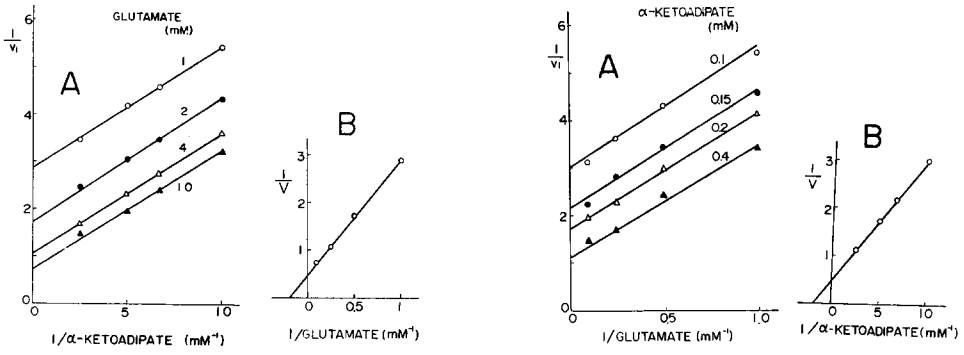


Fig. 3. Effect of α-ketoadipate concentration on reaction rate. Rate measurements were made as described in EXPERIMENTAL PROCEDURE, with 6 μg of Step-4 protein. A. Double reciprocal plots of initial velocity with respect to α-ketoadipate concentration at the fixed concentrations of glutamate indicated on each line. B. Secondary plot of apparent maximum velocities against glutamate.

Fig. 4. Effect of glutamate concentration on reaction rate. A. Double reciprocal plots of initial velocity with respect to glutamate concentration at the fixed concentrations of α-ketoadipate indicated on each line. B. Secondary plot of apparent maximum velocities against α-ketoadipate.

Substrate specificity

It was of interest to determine whether the transamination of α-aminoadipate was catalyzed by a specific enzyme or by other known transaminases. In view of a high activity of aspartate aminotransferase in liver mitochondria, the possibility was considered that the transamination of α-aminoadipate is catalyzed by this enzyme. However, preliminary experiments have shown that the ratios of α-aminoadipate and aspartate aminotransferase activities are different at each step of enzyme purification, suggestive of different enzymes catalyzing these reactions. Chromatography

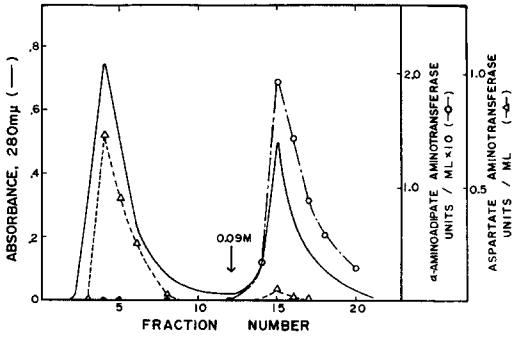


Fig. 5. Chromatographic separation of α-aminoadipate aminotransferase from aspartate aminotransferase on brushite column. The enzyme solution of Step 2 was dialyzed overnight against 0.05 M potassium phosphate buffer (pH 6.8), containing 1 mM EDTA and 8 μM pyridoxal phosphate, and the dialyzed solution containing approx. 20 mg of protein was applied to a column, 2 cm × 3 cm of brushite, which had been equilibrated with 0.05 M potassium phosphate buffer (pH 6.8)–1 mM EDTA–8 μM pyridoxal phosphate. After washing the column with 40 ml of the same buffer, the molarity of the phosphate buffer was increased to 0.09 M. 3.5-ml fractions were collected. α-Aminoadipate aminotransferase activity was measured by Assay 1, and aspartate aminotransferase activity by the method of WADA AND SNELL[11]. One unit of aspartate aminotransferase is the amount of enzyme producing 1 μmole of oxaloacetate at 37°.

of a crude enzyme preparation on brushite or DEAE-cellulose columns effected the separation of the two activities. Fig. 5 illustrates the result of a typical experiment carried out with brushite. Almost all the aspartate aminotransferase activity was eluted with 0.05 M potassium phosphate buffer, while the activity to transaminate α-aminoadipate eluted at 0.09 M concentration. In this connection, it may be added that a crystalline preparation of aspartate aminotransferase obtained from pig heart mitochondria failed to catalyze the transamination between α-aminoadipate and α-ketoglutarate, tested with the amount of enzyme about 100 times greater than that readily catalyzed aspartate-α-ketoglutarate reaction.

TABLE IV

TRANSAMINATION WITH α-KETOGLUTARATE

The transaminase activities were determined by Assay 2, except that L-α-aminoadipate was substituted by amino acids indicated. Step-4 protein (8 μg) was used.

Amino acid	*Relative activity**
L-α-Aminoadipate	100
DL-α-Aminopimelate**	14
L-Norleucine	15
L-Aspartate, L-alanine, L-α-aminobutyrate, glycine, L-valine, L-leucine, L-isoleucine, L-norvaline, L-ε-aminocaproate, L-threonine, L-serine, L-homoserine, L-cysteine, L-methionine, L-lysine, L-arginine, L-citrulline, L-ornithine, L-histidine, L-tyrosine***, L-tryptophan***, L-phenylalanine, L-proline, L-glutamine	<2

* Transaminase activity was expressed in terms of L-α-aminoadipate, which was assigned a value of 100.

** 40 μmoles of the DL-compound were used.

*** Saturated solutions were used.

The ability of a purified preparation of α-aminoadipate aminotransferase to catalyze the transamination between α-ketoglutarate and various amino acids was examined. Table IV shows the relative rate of transamination. As the table shows only α-aminoadipate, α-aminopimelate and norleucine were found to be transaminated at an appreciable rate. Aspartate and alanine were inert as substrates, and this fact rules out significant contaminations of aspartate and alanine aminotransferases. With α-ketoadipate as the amino group acceptor, the aminotransferase seems to utilize only glutamate, α-aminopimelate and norleucine as the amino donors. High-voltage paper electrophoresis of the reaction mixture revealed a significant formation of α-aminoadipate only with these amino acids. D-Glutamate was not active as the substrate. Furthermore, those amino acids which were inert as substrates (Table IV) failed to inhibit α-ketoadipate–glutamate reaction, when tested at 0.1-M concentrations (for tyrosine and tryptophan, saturated solutions were used).

DISCUSSION

α-Aminoadipate occurs, in mammalian tissues, as an essential intermediate of lysine catabolism[1]. Recent investigations have shown that lysine was converted to α-aminoadipate *via* saccharopine and also provided evidence that this transformation was achieved in the mitochondrion[2–5]. The present investigation shows that α-aminoadipate is also metabolized to α-ketoadipate by a transamination reaction with α-ketoglutarate in this cell compartment.

α-Aminoadipate aminotransferase purified from rat liver mitochondria behaves kinetically like other transaminases. The results of kinetic analyses are consistent with the mechanism that the overall reaction is a sum of two half-reactions and that the enzymic reaction involves a single binary enzyme–substrate complex. The equilibrium constant of near unity for α-aminoadipate aminotransferase reaction is comparable to those reported for glutamate–alanine and glutamate–aspartate reactions[12,13]. The coenzyme of α-aminoadipate aminotransferase could easily be removed by a simple dialysis against phosphate buffer. As is the case with other transaminases, the enzymic activity was largely restored by addition of either pyridoxal or pyridoxamine phosphate. As shown in Fig. 2, pyridoxal phosphate seems to bind with the apoenzyme more strongly than pyridoxamine phosphate. The ease with which the coenzyme is resolved distinguishes the aminotransferase from aspartate or alanine aminotransferase.

Since α-aminoadipate (α-ketoadipate) is a higher homologue of glutamate (α-ketoglutarate) and glutamate (α-ketoglutarate) participates in almost all transaminases, it may be inferred that the transamination of α-aminoadipate (α-ketoadipate) is catalyzed by known transaminase(s). Because of a high activity of aspartate aminotransferase in the mitochondria and the similarity of the reaction it catalyzes, the identity or nonidentity of α-aminoadipate aminotransferase with aspartate aminotransferase was examined. However, evidence for the nonidentity of these enzymes was obtained early in the study from the fact that these enzymes exhibit different activity ratios during enzyme purification and from their chromatographic separation. Examination of substrate specificity with purified preparations of α-aminoadipate aminotransferase indicated that the enzyme has a strict substrate specificity. Fortunately, the inability of the purified preparation to catalyze transaminations between α-ketoglutarate and amino acids listed in Table IV, with the exceptions of α-aminopimelate and norleucine, rules out a significant contamination of most other transaminases and also indicates that these amino acids do not act as amino group donors in this aminotransferase. The same conclusion was reached with α-ketoadipate as an amino acceptor. Purified preparations of aspartate and leucine aminotransferases are inactive in catalyzing the transamination between α-aminoadipate and α-ketoglutarate[14]. In the biosynthetic pathway of lysine in yeast, α-ketoadipate is transaminated to α-aminoadipate[15,16], and evidence is obtained that this transaminase is probably a different enzyme from aspartate aminotransferase[17]. An enzyme of *Neurospora crassa* catalyzing a reversible transamination between imidazoleacetol phosphate and glutamate is reported to utilize α-aminoadipate, histidine and arginine in place of glutamate[18]. α-Aminoadipate aminotransferase described in this paper seems to have different properties from this enzyme in that the former does not catalyze the transaminations of histidine and arginine. BRAUN-

STEIN[19] reported that α-ketoadipate underwent transamination with alanine by a muscle homogenate. As mentioned above, alanine was inert as the substrate for α-aminoadipate aminotransferase from rat liver mitochondria, and no α-ketoadipate–alanine reaction was observed with a crude mitochondrial extract.

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